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Mechanisms involved in the inhibition of myoblast proliferation and differentiation by myostatin.

Joulia D, Bernardi H, Garandel V, Rabenoelina F, Vernus B, Cabello G.

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Muscle growth results from a set of complex processes including myogenic transcription factor's expression and activity, cell cycle withdrawal, myoblast fusion in myotubes, and acquisition of an apoptosis-resistant phenotype. Myostatin, a member of the TGFbeta family, described as a strong regulator of myogenesis in vivo Nature 387 (1997), 83; FEBS Lett. 474 (2000), 71 is upregulated during in vitro differentiation Biochem. Biophys. Res. Commun. 280 (2001), 561. To improve characterization of myostatin's myogenic influence, we stably transfected vectors expressing myostatin and myostatin antisense in C2C12 myoblasts. Here, we found that myostatin inhibits cell proliferation and differentiation. Our results also indicate that myogenin is an important target of myostatin. In addition, overexpressed but not endogenous myostatin decreases MyoD protein levels and induces changes in its phosphorylation pattern. We also established that myostatin overexpression reduces the frequency of G0/G1-arrested cells during differentiation. Conversely, inhibition of myostatin synthesis leads to enhanced cell cycle withdrawal and consequently stimulates myoblast differentiation. We examined the expression patterns of the pRb, E2F1, p53, and p21 proteins involved in cell cycle withdrawal. We found that myostatin overexpression increases p21 and p53 expression, as it does accumulation of hypophosphorylated Rb. Interestingly, myostatin overexpression strongly reduced low-mitogen-induced apoptosis, whereas antisense expression induced contrary changes. In conclusion, these data show the influence of overexpressed myostatin on myoblast proliferation, differentiation, and apoptosis is extended to endogenous myostatin. Though some differences in overexpression or inhibition of endogenous myostatin were observed, it appears that myogenin and p21 are essential targets of this growth factor.

PMID: 12749855 [PubMed - indexed for MEDLINE]

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Adenovirus-mediated arterial gene therapy for restenosis: problems and perspectives.

Feldman LJ, Tahlil O, Steg PG.

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Restenosis remains the main limitation of interventional cardiology. Restenosis occurs when angioplasty-induced intimal hyperplasia as well as arterial remodelling result in flow-limiting renarrowing of the arterial lumen at the angioplasty site. Intimal hyperplasia is an important candidate for gene therapy since it is related to smooth muscle cell proliferation, which is an inviting target for molecular antiproliferative strategies. To date, adenoviral vectors are, by far, the most efficient vectors to perform in vivo arterial gene transfer. These vectors, as well as others, have been recently used to demonstrate that therapeutic genes encoding cytotoxic (herpes virus thymidine kinase) or cytostatic (hypophosphorylatable Rb, Gax, endothelial nitric oxide synthase) products successfully inhibit smooth muscle cell proliferation and related intimal hyperplasia. Despite substantial progress, major technical issues, including the toxicity of first-generation adenoviral vectors, inefficient transduction of atherosclerotic arteries, and the risk of extra-arterial transfection remain to be addressed before gene therapy is applied to clinical restenosis.

Publication Types:
- Review
- Review, Tutorial

PMID: 9552512 [PubMed - indexed for MEDLINE]

1: Endocr Rev. 1996 Oct;17(5):481-517. Related Articles, Links

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Growth hormone and the insulin-like growth factor system in myogenesis.

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It is very clear that the GH-IGF axis plays a major role in controlling the growth and differentiation of skeletal muscles, as it does virtually all of the tissues in the animal body. One aspect of this control is unquestioned: circulating GH acts on the liver to stimulate expression of the IGF-I and IGFBP3 genes, substantially increasing the levels of these proteins in the circulation. It also seems that GH stimulates expression of IGF-I genes in skeletal muscle, although there are a number of cases in which skeletal muscle IGF-I expression is elevated in the absence of GH. It is substantially less clear that GH acts directly on skeletal muscle to stimulate its growth; the presence of GH receptor mRNA in skeletal muscle is well established, but most investigators have been unsuccessful in demonstrating any specific binding of GH to skeletal muscle or to myoblasts in culture. It has been equally difficult to show direct actions of GH on cultured muscle cells; the only positive report concludes that the early insulin-like effects of GH can result from direct interactions between GH and isolated muscle cells. The effects of the IGFs on skeletal muscle are much clearer. It is well established by studies in a number of laboratories on a variety of systems that IGFs stimulate many anabolic responses in myoblasts, as they do in other cell types. IGFs have the unusual property of stimulating both proliferation and differentiation of myoblasts, responses that are generally believed to be mutually exclusive; in myoblasts, they are in fact temporally separated. The stimulation of differentiation by IGF-I is (at least in part) a result of substantially increased levels of the mRNA for myogenin, the member of the MyoD family most directly associated with terminal myogenesis. As levels of myogenin mRNA rise, those of myf-5 mRNA (the only other member of the MyoD family expressed significantly in L6 myoblasts) fall dramatically, although myf-5 expression is required for the initial elevation of myogenin. The effects of IGFs are significantly modulated by IGFBPs secreted by myoblasts in serum-free medium, inhibitory IG-FBPs-4 and -6 are expressed and secreted by L6A1 myoblasts, while expression of IGFBP-5 rises dramatically as differentiation proceeds. Other myoblasts also secrete IGFBP-2. Even if exogenous IGFs are not added to the low-serum "differentiation" medium, myoblasts express sufficient amounts of autocrine

IGF-II to stimulate myogenesis after a period of time; some myogenic cell lines, (such as Sol 8) are so active in expressing the IGF-II gene that it is not possible to demonstrate effects of exogenous IGFs. This autocrine expression of IGFs is by no means unique to skeletal muscle cells; indeed, it is so widely seen in cells responding to mitogenic stimuli that we suggest that IGFs can be viewed as extracellular second messengers that mediate most, if not all, such actions of agents that stimulate cell proliferation. The component of serum that suppresses IGF-II gene expression under "growth" conditions appears to be the IGFs themselves, which exhibit a very high potency in the feedback inhibition of IGF-II expression. In addition, IGFs have effects on the expression of other genes related to differentiation. Treatment of L6A1 cell with IGFs suppresses their expression of the myogenesis-inhibiting TGF beta s with a time course consistent with an initial proliferative step followed by differentiation, i.e. expression is first increased and then very substantially decreased. It is not established that this plays a role in control of differentiation, but experiments with FGF antisense constructs suggests that this may well be the case. Until recently, IGFs were the only circulating agents known to stimulate myoblast differentiation, in contrast to the relatively large number of growth factors that inhibit the process. It is now clear that thyroid hormones and RA also stimulate myogenesis, and that IL-15 enhances the stimulatory eff

Publication Types:
- Review
- Review, Academic

PMID: 8897022 [PubMed - indexed for MEDLINE]

1: Biochem Biophys Res Commun. 2002 Mar 1;291(3):701-6. Related Articles, Links

Myostatin knockout in mice increases myogenesis and decreases adipogenesis.

Lin J, Arnold HB, Della-Fera MA, Azain MJ, Hartzell DL, Baile CA.

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Growth differentiation factor-8 (GDF-8), or Myostatin, plays an important inhibitory role during muscle development. Since muscle and adipose tissue develop from the same mesenchymal stem cells, we hypothesized that Myostatin gene knockout may cause a switch between myogenesis and adipogenesis. Male and female wild type (WT) and Myostatin knockout (KO) mice were sacrificed at 4, 8, and 12 weeks of age. The gluteus muscle (GM) was larger in KO mice compared to WT mice at 8 ($P < 0.01$) and 12 ($P < 0.001$) weeks. At 12 weeks, KO mice had decreased fat depots ($P < 0.01$). Compared to 12-week-old WT mice, serum leptin concentration in KO mice was lower ($P < 0.001$) and leptin mRNA expression was decreased ($P < 0.01$) in inguinal adipose tissue. CCAAT/enhancer binding protein-alpha (C/EBPalpha) and peroxisome proliferator-activated receptor-gamma (PPARgamma) levels in adipose tissue were significantly lower in KO mice compared to WT mice. Thus, increased muscle development in Myostatin knockout mice is associated with reduced adipogenesis and consequently, decreased leptin secretion. (c)2002 Elsevier Science (USA).

PMID: 11855847 [PubMed - indexed for MEDLINE]

1: Growth Factors. 2001;18(4):251-9. Related Articles, Links

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GDF-8 propeptide binds to GDF-8 and antagonizes biological activity by inhibiting GDF-8 receptor binding.

Thies RS, Chen T, Davies MV, Tomkinson KN, Pearson AA, Shakey QA, Wolfman NM.

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GDF-8 is a new member of the TGF-beta superfamily which appears to be a negative regulator of skeletal muscle mass. Factors which regulate the biological activity of GDF-8 have not yet been identified. However, the biological activities of TGF-beta superfamily members, TGF-beta1, -beta2 and -beta3, can be inhibited by noncovalent association with TGF-beta1, -beta2 and beta3 propeptides cleaved from the amino-termini of the TGF-beta precursor proteins. In contrast, the propeptides of other TGF-beta superfamily members do not appear to be inhibitory. In this investigation, we demonstrate that purified recombinant GDF-8 propeptide associates with purified recombinant GDF-8 to form a noncovalent complex, as evidenced by size exclusion chromatography and chemical crosslinking analysis. Furthermore, we show that GDF-8 propeptide inhibits the biological activity of GDF-8 assayed on A204 rhabdomyosarcoma cells transfected with a (CAGA)12 reporter construct. Finally, we demonstrate that GDF-8 propeptide inhibits specific GDF-8 binding to L6 myoblast cells. Collectively, these data identify the GDF-8 propeptide as an inhibitor of GDF-8 biological activity.

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Transcriptional Effects of Chronic Akt Activation in the Heart*

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Akt activation reduces cardiomyocyte death and induces cardiac hypertrophy. To help identify effector mechanisms, gene expression profiles in hearts from transgenic mice with cardiac-specific expression of activated Akt (myr-Akt) were compared with littermate controls. 40 genes were identified as differentially expressed. Quantitative reverse transcription-PCR confirmed qualitative results of transcript profiling for 9 of 10 genes examined, however, there were notable quantitative discrepancies between the quantitative reverse transcription-PCR and microarray data sets. Interestingly Akt induced significant up-regulation of insulin-like growth factor-binding protein-5 (IGFBP-5), which could contribute to its anti-apoptotic effects in the heart. In addition, Akt-mediated down-regulation of peroxisome proliferator-activated receptor (PPAR) γ coactivator-1 (PGC-1) and PPAR-α may shift myocytes toward glycolytic metabolism shown to preserve cardiomyocyte function and survival during transient ischemia. IGFBP-5 transcripts also increased after adenoviral gene transfer of myr-Akt to cultured cardiomyocytes, suggesting that this represents a direct effect of Akt activation. In contrast, substantial induction of growth differentiation factor-8 (GDF-8), a highly conserved inhibitor of skeletal muscle growth, was observed in transgenic hearts but not after acute Akt activation *in vitro*, suggesting that GDF-8 induction may represent a secondary effect perhaps related to the cardiac hypertrophy seen in these mice. Thus, microarray analysis reveals previously unappreciated Akt regulation of genes that could contribute to the effects of Akt on cardiomyocyte survival, metabolism, and growth.

The serine-threonine kinase Akt (or protein kinase B) has well documented anti-apoptotic effects in many systems (1–3). We have shown that expression of a constitutively active mutant of Akt (myr-Akt) is sufficient to block apoptosis in hypoxic neonatal rat cardiomyocytes *in vitro* (4) and *in vivo* prevents cardiac injury while preserving heart function during ischemia-reperfusion injury (5). The downstream targets of Akt that mediate cell survival in the heart remain poorly characterized. Indeed some Akt substrates (*e.g.* Bad, glycogen synthase kinase-3, and Bcl-2) identified in other cell types appear to be either expressed at very low levels or not phosphorylated by Akt in cardiomyocytes (5, 6). These data suggest that additional Akt-dependent phosphorylation, translation, and/or transcription events may be required for Akt-mediated cytoprotection in the heart.

Translational effects of Akt involve the phosphorylation and activation of the mammalian target of rapamycin (mTOR)[1] that in turn phosphorylates 4E-BP1 and p70S6 kinase (7). The net effect of these phosphorylation events is enhanced translation of specific mRNA subset(s), which is bound by the initiation factor eIF-4F and/or the ribosomal S6 subunit. In contrast, the transcriptional effects of Akt are less well defined, although the importance of these events may be greater than initially realized (8, 9). Akt-regulated gene transcription has been described for Glut-1 (10), vascular endothelial growth factor (11), and Bcl-2 (12), and a number of Akt-regulated transcription factors have been identified. Akt directly phosphorylates Forkhead box transcription factors, class O (FOXOs) (13–15) and may also regulate, through direct and/or indirect mechanisms, AP-1, cAMP-response element-binding protein, and NF-κB (16–19).

To examine the transcriptional effects of Akt in the heart we analyzed the changes in global gene expression in transgenic mice with cardiac-specific expression of myr-Akt using DNA microarrays. This approach enabled the quantitation of the effects of Akt activation on ~11,000 genes. Results of interest were validated by quantitative RT-PCR (QRT-PCR). Here we identify genes differentially regulated by chronic Akt activation in the heart and demonstrate that modulated transcripts represent a combination of primary and secondary effects. The importance of confirming microarray results of interest using additional, complimentary techniques is discussed.

EXPERIMENTAL PROCEDURES

Mice—Generation and phenotypic characterization of myr-Akt mice is described elsewhere in detail (20). In brief, the cDNA encoding hemagglutinin-tagged Akt with a *src* myristoylation (myr) signal (kindly provided by Dr. Thomas F. Franke, Columbia University) was subcloned downstream of the 5.5-kb murine α-myosin heavy chain promoter (generously provided by Dr. Jeffrey Robbins, Division of Molecular Cardiovascular Biology, Cincinnati Children's Hospital Research Foundation) and used to generate transgenic mice through oocyte injection. Positive founders were identified by Southern blotting and bred to wild-type C57BL6 mice for six generations. Two transgenic (TG) lines were maintained; the 20 line exhibited X-linked inheritance, whereas the 564 line exhibited autosomal inheritance. TG-positive F3 mice were used for studies and compared with TG-negative littermates.

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[1] The abbreviations used are: mTOR, mammalian target of rapamycin; FOXO, Forkhead box transcription factor, class O; QRT-PCR, quantitative reverse transcription-PCR; myr, myristoylated; TG, transgenic; AvDiff, average difference; Ad, adenoviral vector; EGFP, enhanced green fluorescent protein; NRVM, neonatal rat ventricular cardiomyocyte; MLC1F/3F, myosin alkali light chain 1 fast/3 fast; OTT, ovary testis transcribed; IGF, insulin-like growth factor; IGFBP, IGF-binding protein; PPAR, peroxisome proliferator-activated receptor; PGC-1, PPAR-γ coactivator-1; GDF-8, growth differentiation factor-8.

Both lines express myr-Akt specifically in the heart at levels 5–7-fold higher than the endogenous molecule and exhibit a substantial increase in Akt activation as measured by both *in vitro* kinase assays and *in vivo* phosphorylation of known substrates (20).

Preparation of cRNA for Microarray Analysis—Total RNA was extracted from F3, 6-week-old, 20 line male mouse hearts using TRIzol (Invitrogen) according to the manufacturer's recommendations. RNA was resuspended in diethyl pyrocarbonate-treated H_2O and further purified using the Qiagen (Chatsworth, CA) RNeasy total RNA isolation kit according to the manufacturer's instructions. RNA was quantified, and samples (n = 2–5 hearts) were pooled such that pooled RNA represented equal amounts (10 μg) of RNA from TG-positive or TG-negative mice within the litter. This was repeated in three independent experiments. Pooled samples (10 μg) were used to generate cDNA using the Superscript Choice system (Invitrogen) according to the Affymetrix protocol (Affymetrix, Santa Clara, CA). Resulting cDNA was used to generate biotin-labeled cRNA using the ENZO Bioarray High Yield transcript labeling kit (Affymetrix). cRNA (20 μg) was fragmented in fragmentation buffer (40 mM Tris (pH 8.1), 100 mM potassium acetate, 30 mM magnesium acetate) for 35 min at 94 °C. The quality of the cRNA was checked by hybridization to Test2 arrays (Affymetrix) according to the manufacturer's instructions. Subsequently samples were hybridized to Affymetrix mU74A microarrays, and bound sequences were identified by staining and scanning according to Affymetrix protocols.

Analysis of Microarray Data—To enable comparison between experiments expression data were globally scaled to an average intensity of 1500 using the Affymetrix Microarray Suite™ software. A minimum value of 150 was assigned to all average differences (AvDiffs) with an intensity measurement below 150. Two parameters, the AvDiff and the absolute call (present or absent), extracted from the Affymetrix data files, were used in the data analysis, which was performed using Genespring™ (Silicon Genetics, CA). Results were sorted using a combination of high and low stringency filtering criteria. High stringency filtering required that a gene should have an absolute call of present in six of six samples with a mean -fold change of $\geq \pm 1.6$. Low stringency filtering required that the gene be called present in two of the three replicates in the more highly expressing group with a mean AvDiff of ≥ 750 and mean -fold change of ≥ 2. Mean -fold changes between groups were calculated from the mean AvDiffs. Data passing these criteria were combined and subjected to statistical analysis.

QRT-PCR Analysis—Total RNA was isolated and purified from the hearts of F3, 6-week-old male mice from the 20 and 564 transgenic lines as described above. Following purification RNA was quantified in triplicate using Ribogreen (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. RNA (5 μg) was treated (10 min at 20 °C) with amplification grade DNase 1 (Invitrogen) following which the DNase 1 was heat-inactivated (5 min at 75 °C). QRT-PCR was performed in duplicate using the Brilliant One-Step QRT-PCR kit (Stratagene, La Jolla, CA) containing SYBR Green I (1:30,000, Sigma), forward and reverse primers (50 nM each), and sample RNA (90 ng). Primers were designed to be compatible with a single QRT-PCR thermal profile (48 °C for 30 min, 95 °C for 10 min, and 40 cycles of 95 °C for 30 s and 60 °C for 1 min) such that multiple transcripts could be analyzed simultaneously. Accumulation of PCR product was monitored in real time (Mx4000, Stratagene), and the crossing threshold (Ct) was determined using the Mx4000 software. For each set of primers, a no template control and a no reverse amplification control were included. Postamplification dissociation curves were performed to verify the presence of a single amplification product in the absence of DNA contamination. -Fold changes in gene expression were determined using the ΔCt method with normalization to total RNA (21, 22).

Adenoviral Vectors (Ads)—Ad·EGFP·β-gal contains cytomegalovirus-driven expression cassettes for β-galactosidase and enhanced green fluorescent protein (EGFP) (5). Ad·Akt(AA) utilizes a similar viral backbone but encodes a dominant-negative Akt mutant and was kindly provided by Dr. Wataru Ogawa, Kobe University, Japan (23). Ad·myr-Akt and Ad·EGFP mediate expression of hemagglutinin-tagged constitutively active Akt or EGFP, respectively, and have been described previously (5). Ads were amplified in 293 cells, the particle count was estimated from A_{260}, and the titer was determined by plaque assay. Wild-type adenovirus contamination was excluded by the absence of PCR-detectable early region 1 (E1) sequences.

In Vitro Studies of myr-Akt Expression—Primary cultures of neonatal rat ventricular cardiomyocytes (NRVMs) were prepared from the cardiac ventricles of Sprague-Dawley neonates as described previously (5). To study the effects of transient transgene expression, myocytes were infected with adenoviral vectors at a multiplicity of infection of 100 for 24 h in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cells were subsequently serum-starved for 24 h prior to RNA extraction. RNA was extracted, purified, and quantified as described above.

Immunoblotting—Hearts from littermate control and myr-Akt-expressing mice were removed from deeply anesthetized animals, snap frozen, and crushed under liquid nitrogen before tissue was homogenized in cold lysis buffer (20 mM Tris-HCl (pH7.6), 150 mM NaCl, 1% Triton X-100, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 μg/ml aprotinin). Proteins from NRVMs were extracted by scraping cells directly into cold lysis buffer as described previously (4). Protein concentration was measured by the Bradford method (Bio-Rad). Proteins (30 μg) were separated by SDS-PAGE on 12% separation gels and transferred to nitrocellulose membranes (Schleicher & Schuell) by semidry transfer. Blots were incubated with anti-Akt (1:1000, Cell Signaling) overnight at 4 °C and subsequently incubated with horseradish peroxidase-conjugated secondary antibody (1:5000, Dako). Immunoreactive bands were detected by enhanced chemiluminescence (Cell Signaling).

Statistics—Data are represented as mean ± S.E. Data were compared by two-tailed Student's t test. The null hypothesis was rejected for $p < 0.05$.

RESULTS

Effects of myr-Akt Expression on Gene Expression in the Heart—To identify genes differentially regulated by Akt in the heart we examined the gene expression profiles of mice with cardiac-specific expression of myr-Akt (20 line) compared with TG-negative littermate controls. The experiment was repeated three times to reduce erroneous data that can arise when pooled RNA alone is used as a substitute for experimental replication (25). Genes of interest were identified using the described filtering protocols and examined for statistically significant differences in expression. These analyses revealed that expression of myr-Akt in the heart resulted in the differential regulation of 40 (21 up-regulated and 19 down-regulated) of the ~11,000 genes examined (Tables I and II).

It is surprising to observe that the two genes with the greatest -fold changes in expression are not usually expressed in the heart. Myosin alkali light chain 1 fast/3 fast (MLC1F/3F, up-regulated 11.8-fold) is predominantly expressed in skeletal muscle (26) and the ovary testis transcribed (OTT, up-regulated 11.1-fold) gene is usually only expressed in the ovary or the testis (27). Induction of insulin-like growth factor-binding protein-5 (IGFBP-5) by insulin-like growth factor-I (IGF-I) via phosphatidylinositol 3-kinase and mTOR has been observed previously (28), although a direct connection to Akt has not been reported. Some genes of related function were coordinately regulated by chronic Akt expression. For instance, the potent inhibitor of angiogenesis pigment epithelium-derived factor was up-regulated 2.6-fold, while the angiogenic factor vascular endothelial growth factor was down-regulated 1.8-fold. In addition, transcripts for peroxisome proliferator-activated receptor α (PPAR-α) and peroxisome proliferator-activated receptor γ coactivator-1 (PGC-1), both involved in fatty acid metabolism, were down-regulated.

Validation of Microarray Data for myr-Akt-expressing Mice by QRT-PCR—The differential expression of six up-regulated and four down-regulated genes, identified by microarray analysis, were validated by QRT-PCR. Relative transcript levels were determined in F3, 20 line TG-positive males compared with TG-negative male littermate controls (Fig. 1). QRT-PCR analysis confirmed 7 of the 10 genes were statistically differentially regulated ($p < 0.05$) in the 20 line. Cardiac ankyrin repeat protein, pigment epithelium-derived factor, and IGF-II, although differentially regulated in accordance with microarray data, did not achieve statistical significance. Cardiac ankyrin repeat protein and pigment epithelium-derived factor were subsequently confirmed as differentially regulated ($p < 0.05$) in the 564 line. Although the -fold change of some genes (IGFBP-5, pigment epithelium-derived factor, PGC-1, PPAR,

TABLE I
Genes significantly up-regulated in myr-Akt-expressing mice

Genes identified as up-regulated by microarrays were filtered and analyzed as described. SNAP, soluble *N*-ethylmaleimide-sensitive factor attachment protein; EST, expressed sequence tag.

Gene name	-Fold change	*p*	GenBank™
MLC1F/MLC3F	11.8	<0.01	X12973
Ovary testis transcribed	11.1	<0.01	X96603
Insulin-like growth factor-binding protein-5	5.4	<0.05	L12447
Growth differentiation factor-8	5.1	<0.01	U84005
FXYD ion transport regulator 5	3.6	<0.05	U72680
Procollagen, type VIII, α1	2.8	<0.05	X66976
Lysozyme P	2.8	<0.01	X51547
Golgi SNAP receptor complex member 2	2.7	<0.05	AI847904
Pigment epithelium-derived factor	2.6	<0.05	AF036164
Cardiac ankyrin repeat protein	2.5	<0.01	AF041847
Receptor activity modifying protein 1	2.1	<0.05	AJ250489
Complement component 1qc	2.1	<0.01	X66295
Peroxisomal biogenesis factor 11a	2.0	<0.05	AF093669
Odorant-binding protein Ib	2.0	<0.05	AW046850
Ia-associated invariant chain	1.9	<0.05	X00496
Heterogeneous nuclear ribonucleoprotein L	1.6	<0.01	AB009392
Procollagen C-proteinase enhancer protein	1.6	<0.05	X57337
4 ESTs	(3.0–1.8)	<0.05	

TABLE II
Genes significantly down-regulated in myr-Akt-expressing mice

Genes identified as down-regulated by microarrays were filtered and analyzed as described. EST, expressed sequence tag.

Gene name	-Fold change	*p*	GenBank™
Aryl-hydrocarbon receptor-interacting protein	5.0	<0.05	AW227620
Matrin 3	3.0	<0.01	AB009275
PGC-1	2.9	<0.01	AF049330
Short stature homeobox 2	2.4	<0.05	U66918
Homeodomain-interacting protein kinase 3	2.3	<0.05	AF077660
Esterase 1	2.2	<0.02	AW226939
Cd27-binding protein (SIVA)	2.1	<0.05	AF033115
Insulin-like growth factor II	2.1	<0.05	X71922
Vascular endothelial growth factor	1.8	<0.05	M95200
PPAR-α	1.7	<0.05	X57638
Methylmalonyl-coenzyme A mutase	1.6	<0.05	X51941
8 ESTs	(1.8–3.7)	<0.05	

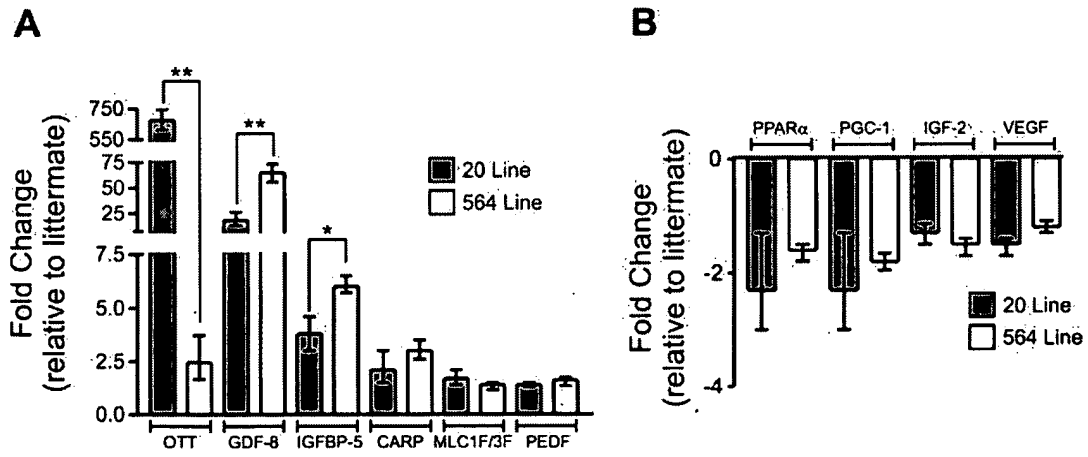

FIG. 1. **Comparison of gene expression of sequences identified as differentially regulated by microarray analysis in two myr-Akt-expressing lines.** 10 genes identified as differentially regulated in 20 line transgenic hearts by microarray analysis were examined in two myr-Akt-expressing lines (20 line and 564 line) by QRT-PCR using gene-specific primers. Amplified products were detected in real time using SYBR Green I, and product specificity was confirmed by postamplification dissociation curve analysis. Gene expression levels in TG20 and TG564 transgenic hearts were determined relative to littermate controls (n = 3–4 in both groups). *A*, up-regulated genes: relative expression levels of six up-regulated genes in the 20 line and 564 line myr-Akt-expressing mice. Data are expressed as mean ± S.E. (*, $p < 0.05$; **, $p < 0.01$). *B*, down-regulated genes: relative expression levels of four down-regulated genes in the 20 line and 564 line myr-Akt-expressing mice. *VEGF*, vascular endothelial growth factor; *PEDF*, pigment epithelium-derived factor; *CARP*, cardiac ankyrin repeat protein.

and vascular endothelial growth factor), as determined by QRT-PCR analysis, correlated with the -fold change reported by microarray analysis there were three major discrepancies. The greatest discrepancy was observed in the expression levels of OTT, which was reported as 11.1-fold up-regulated by microarray analysis compared with 675-fold by QRT-PCR (Table I and Figs. 1 and 2). The second major discrepancy was seen in the expression levels of growth differentiation factor-8 (GDF-8), which was reported as 5.1-fold up-regulated in TG20-positive hearts by microarray analysis compared with 18.4-fold

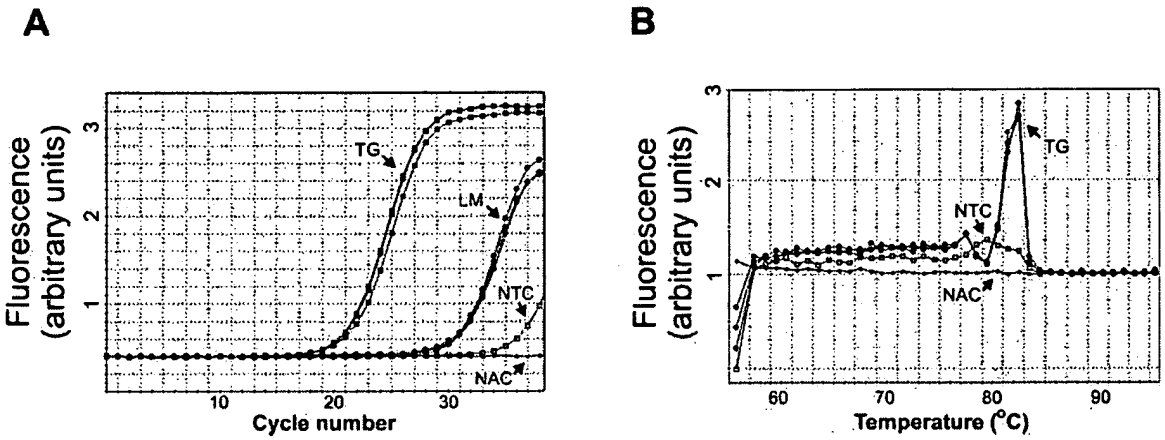

FIG. 2. **Amplification curves and postamplification dissociation curves for OTT in 20 line mice.** Total RNA was prepared from 20 line TG and littermate (*LM*) controls and subjected to QRT-PCR analysis of OTT mRNA levels using gene-specific primers and postamplification melt curve analysis. A no template control (*NTC*) and a no amplification control (*NAC*) were included to confirm accumulation of a single PCR product of the predicted melting temperature in the absence of DNA contamination. *A*, amplification: amplified product was detected after an average of 18.7 cycles of PCR in TG hearts compared with an average of 28.1 cycles in littermate control hearts ($n = 3$ in both groups). Accumulation of nonspecific product was observed in the no template control after 33 cycles. No amplification was observed in the no amplification control confirming the absence of DNA contamination. *B*, melting point analysis: the first derivative of the postamplification dissociation curve demonstrates that the accumulated product has a single melting point in accordance with that predicted for the specific OTT amplicon. Minimal nonspecific primer-dimer was observed in the no template control, and no DNA-derived product was observed in the no amplification control.

up-regulated by QRT-PCR. These discrepancies may be explained, in part, by the greater dynamic range afforded by QRT-PCR analysis. However, this explanation cannot account for the difference between microarray and QRT-PCR data for MLC1F/3F expression. An 11.8-fold ($p < 0.01$) up-regulation of MLC1F/3F was recorded by microarray analysis compared with a 1.7-fold ($p < 0.05$) up-regulation as determined by QRT-PCR. The relative expression of MLC1F/3F was further examined by Northern blot analysis, which revealed a modest increase in MLC1F/3F mRNA levels in TG-positive hearts in accordance with the QRT-PCR data and in deference to the microarray data (data not shown).

Comparison of Differential Gene Expression between Two myr-Akt TG Lines—To control for differences in transgene insertion, expression, and activity, we determined the relative expression of the 10 genes examined by QRT-PCR in the 20 line in a second myr-Akt-expressing line, the 564 line (Fig. 1). For all genes except OTT, the pattern of differential expression observed in TG20 mice was confirmed in TG564 mice, although the -fold change in expression was significantly greater in the 564 line for GDF-8 and IGFBP-5 (64.9 *versus* 18.4, $p < 0.01$ and 6.0 *versus* 3.8, $p < 0.05$, respectively; Fig. 1*A*). Although OTT mRNA was detected in the TG564 hearts, there was no difference in the low level of expression between TG-positive and -negative littermates.

Effects of Transient myr-Akt Expression on IGFBP-5 and GDF-8 Transcript Levels in Vitro—We next examined whether IGFBP-5 and/or GDF-8 were directly regulated by acute Akt activation in cardiomyocytes using an *in vitro* system (4). NRVMs were infected with Ad·EGFP, Ad·myr-Akt, or dominant-negative Ad·Akt(AA). Ad·Akt(AA) served as a full-length control for the Akt molecule, including the pleckstrin homology domain but lacking catalytic activity. The effects of these constructs on IGFBP-5 and GDF-8 gene expression were determined by QRT-PCR (Fig. 3*A*). Expression of Ad·myr-Akt, at levels comparable to those observed in the TG mice (Fig. 3*B*), significantly up-regulated IGFBP-5 (7.2-fold, $p < 0.05$) compared with Ad·Akt(AA). This finding corroborates a previous study in vascular smooth muscle cells that demonstrated IGFBP-5 mRNA up-regulation by IGF-I in a phosphatidylinositol 3-kinase/mTOR-dependent manner (28). In contrast,

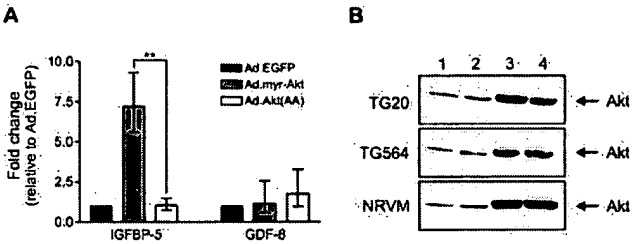

FIG. 3. **Transient expression of myr-Akt increases mRNA encoding IGFBP-5 but not that of GDF-8.** NRVMs were infected with Ad·EGFP, Ad·myr-Akt, or Ad·Akt(AA) (multiplicity of infection = 100 for all), and total RNA or protein was extracted after 24 h in serum-free medium. *A*, expression of IGFBP-5 and GDF-8 mRNA: relative expression levels of IGFBP-5 and GDF-8 were determined by QRT-PCR using gene-specific primers. Ad·myr-Akt increased the expression of IGFBP-5 by 7.2-fold relative to Ad·EGFP, whereas Ad·Akt(AA) did not. In contrast, Ad·myr-Akt had no effect on expression levels of GDF-8. Data are expressed as mean ± S.E. (**, $p < 0.01$; $n = 3$ in all groups). *B*, immunoblots of myr-Akt expression *in vivo* and *in vitro*: the expression levels of myr-Akt and endogenous Akt were determined to validate the comparison between *in vivo* and *in vitro* QRT-PCR data. Proteins (30 μg) from hearts or cultured NRVMs were separated by SDS-PAGE, and Akt expression was determined by immunoblotting. *Top panel*, 20 line littermate controls (*lanes 1* and *2*) and TG positives (*lanes 3* and *4*). *Middle panel*, 564 line littermate controls (*lanes 1* and *2*) and TG positives (*lanes 3* and *4*). *Bottom panel*, uninfected NRVMs (*lanes 1* and *2*) and NRVMs infected with Ad·myr-Akt (multiplicity of infection = 100) for 24 h (*lanes 3* and *4*).

Ad·myr-Akt did not alter the expression level of GDF-8 at 24 h and had no effect on GDF-8 expression at either 48 or 72 h (data not shown).

DISCUSSION

Akt protects the heart from ischemia-reperfusion injury (5, 29), although it does not appear to phosphorylate many of its potential downstream targets, including Bad, when expressed in neonatal or adult cardiomyocytes (5). Thus, the mechanisms of Akt cardioprotection remain incompletely defined and may include transcriptional effects. The recent identification of Akt-dependent transcripts (*e.g.* Glut-1, Bcl-2, and Fas ligand) (10–12) and Akt-modulated transcription factors (*e.g.* FOXOs, AP-1, and cAMP-response element-binding protein) (13–17), which

are expressed in the heart, supports this hypothesis. We characterized the transcriptional effects of myr-Akt expression in the heart using DNA microarrays.

It has been suggested that DNA microarray experiments should be repeated with at least three replicates (25) and that the resulting data sets should be filtered and validated to minimize erroneous data. Indeed, as much as one-third of the variation seen during an experimental comparison may be attributable to variations intrinsic to the arrays themselves (30). However, data filters should be used with caution as they can increase the number of false negative results. Thus changes in important, low copy transcripts, which are excluded from analysis by virtue of their low AvDiffs and/or their increased propensity to be called "absent," may be missed. We observed significant changes in the expression of 40 (~0.4%) of the genes examined in myr-Akt-expressing hearts (Tables I and II). Of note, the two transcripts with the greatest -fold changes, OTT and GDF-8, were in the group of genes identified using the "low stringency" filter. This finding illustrates how potentially important data may be missed if too stringent a filter is applied to microarray data sets.

We have demonstrated that Akt activation increases the transcription of IGFBP-5 in the heart. IGFBP-5 may have direct and/or indirect anti-apoptotic activity (31–34). Therefore, IGFBP-5 up-regulation, in an Akt-dependent manner, may be of particular importance to the cardioprotective effects of Akt. In the light of previous studies, Akt-dependent IGFBP-5 up-regulation in the heart is likely to be mediated through mTOR (28). It is therefore interesting to note that rapamycin, an mTOR inhibitor, can dramatically attenuate the protective effects of insulin, which activates Akt, in the heart (29). In this study, we have also shown that Akt down-regulates PGC-1 and PPAR-α in the heart. This may shift cardiomyocyte metabolism away from fatty acid metabolism in favor of glycolysis, which has been shown to protect cardiomyocytes during transient ischemia (35, 36).

Confirmation of microarray data by a previously validated and established technique should be performed for a selection of differentially regulated genes and in particular for genes of specific interest. Of the 10 genes analyzed by QRT-PCR, nine were confirmed in one or both of the transgenic lines as significantly differentially expressed in keeping with the microarray data. However, the degree of differential regulation of OTT, GDF-8, and MLC1F/3F determined by QRT-PCR differed markedly from microarray results (Tables I and II and Fig. 1). OTT mRNA has been described only in the testis and ovary (27), and it was initially unclear why this gene should be up-regulated by Akt activation in the heart. As the inheritance in the 20 line is X-linked and OTT is encoded on the X chromosome (27), we hypothesize that the up-regulation of OTT may be an insertional effect of the transgene construct. Consistent with this hypothesis, OTT was not differentially regulated in the 564 line in which the low level of expression was similar to that seen in transgene-negative littermates from both lines and wild-type controls (data not shown). The possibility that the discrepancy between the two lines represents an insertional effect on an autosome in the TG564 mice (for example in a *trans*-acting element regulating OTT expression) appears less likely but has not been formally excluded. As microarray characterization of transgenic mice becomes more common and the murine physical map better characterized, the hitherto latent frequency of insertional events may become more apparent.

The disparity between microarray and QRT-PCR data for the expression levels of GDF-8 and MLC1F/3F highlights two other important limitations of microarray data: dynamic range and sequence specificity. The -fold change in expression of GDF-8 in TG20 hearts, compared with littermate controls, was reported as 5.1-fold up-regulated by microarray analysis. In contrast, analysis of GDF-8 expression in the 20 line by QRT-PCR, likely a more accurate means of quantifying mRNA levels, revealed that GDF-8 was up-regulated by 18.4-fold. This underestimation of -fold change was even greater for OTT, which was found to be 11.1-fold up-regulated by microarray analysis compared with 675-fold by QRT-PCR (Table I and Figs. 1 and 2). The problem of false positive results reported by microarray analysis was illustrated by the MLC1F/3F data, reported as 11.8-fold up-regulated by microarray analysis compared with 1.7-fold (20 line) and 1.4-fold (564 line) by QRT-PCR (Table I and Fig. 1). This false positive result could reflect an error in the sequences on the microarray, the occurrence of which was dramatically demonstrated when up to one-third of the sequences on one set of mouse arrays were found to be wrong (37). Other possibilities for this type of error include cross-hybridization by splice variants, related genes, and/or pseudogenes.

The Akt/mTOR pathway has been identified as the crucial regulator of skeletal muscle and pancreatic islet cell hypertrophy *in vivo* (38, 39). In both our myr-Akt-expressing mouse lines cardiac hypertrophy, with no evidence of decompensation, was observed at 6 weeks (20). Akt therefore promotes both skeletal and cardiac muscle hypertrophy. As Akt promotes cardiac hypertrophy, we hypothesize that the observed up-regulation of GDF-8, a negative regulator of muscle growth, acts as part of a negative feedback loop limiting heart size. The phenomenon of negative feedback and activation of adaptive mechanisms is recognized but infrequently described in transgenic and knockout mice (40, 41). GDF-8, also termed myostatin, is highly conserved across species, and although first characterized in skeletal muscle (42, 43) it has also been identified in the heart (44). The hypothesis that GDF-8 up-regulation is a secondary event is supported by our *in vitro* experiments where expression of myr-Akt, at levels similar to those seen in TG mice (Fig. 3*B*), resulted in the up-regulation of IGFBP-5 but not GDF-8 (Fig. 3*A*). It remains unclear whether GDF-8 expression is related to myocyte size or organ mass (24).

In summary, these data demonstrate that chronic Akt activation results in the differential regulation of ≥40 genes in the heart. Several of the observed changes generate intriguing hypotheses regarding the effects of Akt in the heart and possible mechanisms underlying Akt-mediated cardioprotection. Akt-dependent up-regulation of the anti-apoptotic molecule IGFBP-5 may be of particular importance and could contribute to the observed cytoprotective effects of Akt in the heart. Similarly Akt down-regulation of PGC-1 and PPAR-α could shift myocytes toward glycolytic metabolism previously shown to help preserve cardiomyocyte function and survival during transient ischemia (35, 36). Chronic Akt activation in the heart was associated with the differential regulation of a subset of genes that are dissimilar to those observed with acute Akt activation in other cell types, emphasizing the tissue and temporal specificity of changes in transcription profiles (9). In the myr-Akt mice, some changes (*e.g.* IGFBP-5) appear to be direct consequences of Akt activation and were recapitulated in cardiomyocytes *in vitro*, while other transcripts (*e.g.* GDF-8) were not induced by acute Akt activation *in vitro* and therefore likely represent an indirect effect of the transgene. Given the role of GDF-8 in limiting skeletal muscle growth, we hypothesize that the dramatic up-regulation of GDF-8 observed in hypertrophied hearts may represent a negative feedback mechanism. However, additional studies will be necessary to demonstrate the functional relevance of the observed alterations in transcript levels. Finally, while our transcript profiling and QRT-

PCR data were generally concordant, there were some striking discrepancies in the quantitative assessment of mRNA changes, underscoring the importance of validation of DNA microarray results through additional independent techniques.

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